Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 12043–12047, December 1992
Medical Sciences

Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: A model for diabetic and aging complications

(atherosclerosis/diabetes/aminoguanidine/nitric oxide)

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Contributed by Anthony Cerami, August 31, 1992

ABSTRACT **Advanced glycosylation end products (AGEs) have been implicated in many of the complications of diabetes and normal aging. Markedly elevated vascular tissue and circulating AGEs were linked recently to the accelerated vasculopathy of end-stage diabetic renal disease. To determine the pathogenic role of AGEs *in vivo*, AGE-modified albumin was administered to healthy nondiabetic rats and rabbits alone or in combination with the AGE-crosslink inhibitor aminoguanidine. Within 2–4 weeks of AGE treatment, the AGE content of aortic tissue samples rose to six times the amount found in controls ($P < 0.001$). Cotreatment with aminoguanidine limited tissue AGE accumulation to levels two times that of control. AGE administration was associated with a significant increase in vascular permeability, as assessed by ^{125}I label tracer methods. This alteration was absent in animals that received aminoguanidine in addition to AGE. Significant mononuclear cell migratory activity was observed in subendothelial and periarteriolar spaces in various tissues from AGE-treated rats compared to normal cellularity noted in tissues from animals treated with aminoguanidine. Blood pressure studies of AGE-treated rats and rabbits revealed markedly defective vasodilatory responses to acetylcholine and nitroglycerin compared to controls ($P < 0.001$), consistent with marked NO· inactivation; aminoguanidine treatment significantly prevented this defect. These *in vivo* data demonstrate directly that AGEs, independent of metabolic or genetic factors, can induce complex vascular alterations resembling those seen in diabetes or aging. AGE administration represents an animal model system for the study of diabetic and aging complications as well as for assessing the efficacy of newly emerging therapies aimed at inhibiting advanced glycosylation.**

Despite medical advances in the early diagnosis and treatment of diabetes mellitus, patients continue to develop a number of severe complications, most commonly small and large vessel disease (1, 2). It is now established that glucose reacts spontaneously with the amino groups of a wide range of proteins to form Schiff bases, which, through multiple rearrangements, are transformed into irreversibly bound, chemically reactive adducts termed advanced glycosylation end products (AGEs). These accumulate on vascular wall collagen and basement membrane proteins as a function of age and glycemia (1, 3–5). Many studies have suggested a causal relationship between AGE accumulation and the development of diabetic complications; in this regard, protein crosslinking and trapping reactions as well as AGE ligand–receptor interactions may play a role (3, 5, 6).

The *in vivo* significance of elevated tissue and circulating AGEs as direct contributors to the accelerated vascular disease of diabetes has been emphasized further by *in vitro* studies that have revealed diverse biological activities of AGEs. These, among others, include the induction of endothelial cell permeability (6), monocytic chemotactic activity (7), and an association of AGE–ligand–receptor binding with macrophage activation and cytokine/growth factor secretion (7–9). In addition, AGEs have been implicated in defective nitric oxide (NO·)-dependent vasodilation, a deficit that is prominent in diabetes, and may be due in part to the direct chemical inactivation of endothelium-derived relaxing factor (EDRF) by AGEs accumulated in the vascular endothelium and basement membranes (10). These findings are consistent with vascular perturbations present in various stages of diabetes and/or aging-related vasculopathy, including increased permeability (11), arterio- and atherosclerotic changes (12), and vasodilatory defects (13). The "AGE hypothesis" has been strengthened recently in studies of the specific AGE inhibitor, aminoguanidine (1, 14), shown to prevent several of the complications of diabetes in a number of experimental animal models (15, 16); however, a direct link between AGEs and pathology has not been made.

In the present communication, we report that the short-term administration of exogenous AGE-modified proteins to normal rats or rabbits leads to the covalent attachment of AGEs within the extracellular matrix in a number of tissues. This *de novo* implantation of AGEs within the otherwise normal vasculature is accompanied by a spectrum of vascular abnormalities similar to those observed in chronic diabetes, but in the absence of actual hyperglycemia. Thus, in the present model system, AGE accumulation in vascular tissues and circulation can be linked directly to the pathogenesis of diabetic vascular disease and to the rapid deterioration of vascular function of diabetes-related renal failure.

METHODS

Preparation of Advanced Glycosylation Products. Rat or rabbit serum albumin (RSA) (Sigma) was purified by passage over an Affi-Gel blue column (Bio-Rad) and a heparin-Sepharose CL-6B column (Pharmacia) to remove contaminating serum factors (17). Albumin-derived advanced glycosylation products were prepared as described (10, 17). The formation of AGE was assessed by characteristic fluorescence spectra (10), and AGE levels were quantified by an AGE-specific ELISA (18) [AGE-rat albumin: 62 AGE units/mg, unmodified rat albumin: 0.37 AGE unit/mg; AGE-rabbit

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Abbreviations: AGE, advanced glycosylation end product; EDRF, endothelium-derived relaxing factor; NO·, nitric oxide; ESRD, end-stage renal disease; RSA, rat or rabbit serum albumin; BSA, bovine serum albumin; TBIR, tissue-to-blood isotope ratio; ACh, acetylcholine; NTG, nitroglycerin.

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albumin: 59 AGE units/mg, unmodified albumin: 0.5 unit/mg]. In some instances, AGE-modified or native albumin was subjected to enzymatic digestion with proteinase K (Sigma; 1:100, wt/wt). All reagents were prepared under endotoxin-free conditions and were passed over an endotoxin-binding polymyxin column (Detoxi-gel; Pierce). Prior to *in vivo* administration, each preparation was tested by the *Limulus* amoebocyte lysate assay (E-Toxate, Sigma) and found to contain <0.2 ng of endotoxin per ml.

Animal Studies. Male Lewis rats (150 g), aged 8 wk ($n = 50$) (Charles River Breeding Laboratories), and male New Zealand White rabbits (Hazelton Biologics, Denver, PA) (2.0 kg), aged 4 mo ($n = 16$), were used in these studies. After a 1-wk adaptation period, rats were given tail vein injections with either AGE-modified or native RSA (100 mg/kg per day) or AGE-RSA followed immediately by i.v. injection of aminoguanidine hydrochloride (100 mg/kg per day) for 2–4 wk. Seven days after the injection period, and prior to initiating of further studies, serum AGE levels were determined by ELISA (18). These measurements showed the following: AGE-treated (mean ± SEM), 99.6 ± 15.6 AGE units/ml; native-albumin treated, 19.2 ± 6.1 units/ml; controls, 14.6 ± 4.9 AGE units/ml; AGE plus aminoguanidine hydrochloride, 29.3 ± 8.4 units/ml. Rabbits were subjected to identical treatment protocols, except that they were injected in the marginal ear vein with AGE-modified or native rabbit albumin at a dose of 100 mg/kg per day, with or without aminoguanidine hydrochloride (100 mg/kg per day) for 2 mo.

Vascular Tissue AGE Determination. The nature of the interaction of the exogenous injected ligands with native tissues was initially studied using either ^{125}I-labeled bovine serum albumin (^{125}I-BSA) or ^{125}I-labeled AGE-BSA (^{125}I-AGE-BSA) (50 μg, specific activity = 1.3×10^6 cpm/μg after iodination with carrier-free ^{125}I) administered to rats ($n = 3$ per group) in a single i.v. bolus with or without an injection of aminoguanidine (50 mg). At 1 hr the animals were sacrificed, and the hearts were removed, homogenized in phosphate-buffered saline (PBS), and assayed for total radioactivity. After five washes in PBS, insoluble pellets were extracted an additional five times with increasing concentrations of Triton X-100 (0.1%, 1.0%, 2.0%, 5.0%, 10.0% in PBS). The radioactivity associated with the insoluble material was monitored at each step. Following detergent extraction, insoluble radioactivity associated with ^{125}I-AGE-BSA comprised about 70% of the total heart-associated label, whereas only 25% of the radioactivity after ^{125}I-BSA injection was insoluble. In the presence of the AGE-crosslinking inhibitor aminoguanidine, the postextraction label due to ^{125}I-AGE-BSA was reduced to 35% of the total.

To quantitate the amount of AGE deposited within the main vascular tissues, animals ($n = 5$ per group) were sacrificed by exposure to CO_2; aortic sections were rapidly removed and homogenized, delipidated with acetone and chloroform (1:1), and digested with 1:100 (wt/wt) type VII collagenase (Sigma) for 48 hr at 37°C; and collagen content was determined as described (19). AGE levels were determined by ELISA (18).

***In Vivo* Permeability Studies using Radioactive Tracers.** In vascular permeability studies, ^{125}I-BSA (type V, Sigma) was used as a radiotracer (specific activity = 2.0×10^6 cpm/mg). To correct for blood-associated radioactivity in tissue samples, freshly drawn rat erythrocytes (RBCs) were labeled with ^{51}Cr (^{51}Cr-RBCs) [1 mCi/ml of packed RBCs (1 Ci = 37 GBq)] as described (11). At the indicated intervals after initiation of the injection protocols, rats ($n = 5$ per group) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Five minutes prior to time zero, 0.3 ml of ^{51}Cr-RBCs was injected into the femoral vein. At time zero, 10 mg of tracer ^{125}I-BSA (2.0×10^6 cpm/mg) was injected. At 20 min, aliquots (0.5 ml) of blood were drawn from the carotid artery for radioactivity determination. After removing various organs, tissues were processed for determination of tracer content as described (20). As an index of tracer permeation of tissues, the previously defined term, tissue-to-blood isotope ratio (TBIR), was used (11, 20, 21). TBIR = ^{125}I/^{51}Cr in tissue divided by the ^{125}I/^{51}Cr in blood. A ratio of 1.0 indicates no difference in tissue concentration of ^{125}I from the blood. To obtain an estimate of altered tissue permeability to the labeled tracer, TBIR of each group treated with any of the ligands was compared with that of untreated normal controls. Since the tissues of rats treated with native unmodified ligand yielded a ratio close to 1.0, the TBIR of all other groups was compared to that of rats treated with unmodified albumin as baseline.

***In Vivo* Mononuclear Cell Migratory Activity.** Following perfusion with 10% formaldehyde by standard procedures (22), paraffin-embedded tissue sections from all animal groups ($n = 5$ per group) were processed in triplicate for routine histological examination after staining with Harris' alum hematoxylin stain (23). The presence of monocytes/macrophages in tissues was determined by using the macrophage-specific monoclonal antibody ED2 (Serotec) as primary antibody and biotinylated goat anti-mouse IgG [F(ab')$_2$] (Cappel Research Products) as secondary antibody (23) in 7- to 10-μm sections of frozen tissues.

Assessment of NO·-Dependent Vasodilatory Responses. Lewis rats ($n = 8$ per group) were anesthetized with Nembutal (50 mg/kg, i.p.). Dose–response curves for acetylcholine (ACh) and nitroglycerin (NTG) were generated by injecting drugs directly into the ascending aorta at a constant volume of 1 ml/kg as described (10). Duplicate measurements for each drug dose were obtained for each animal. Aminoguanidine treatment alone was not observed to affect baseline blood pressure or heart rate. Rabbits ($n = 4$ per group) were similarly anesthetized, and blood pressure responses were obtained by cannulation of the central ear artery.

RESULTS

To establish whether exogenously administered AGEs can react covalently with native extracellular matrix proteins, preliminary analyses were carried out using radiolabeled AGE-ligands. Heart muscle from rats injected with a single dose of ^{125}I-AGE- or nonmodified ^{125}I albumin, with or without cotreatment with aminoguanidine, was analyzed for content of covalently bound AGEs, as described in *Methods*. It was noted that after extensive extraction with detergent to remove unreacted AGEs, ≈70% of the injected ^{125}I-AGE-ligand remained associated with the insoluble tissues, consistent with irreversible binding; this was reduced to 35% by the concomitant administration of aminoguanidine.

To determine whether the daily administration of species-matched proteins modified by AGEs leads to significant AGE deposition within vascular tissues, the amount of tissue-associated AGE products was assessed in rat and rabbit aortas after a standard extraction procedure by an AGE-specific ELISA (18). After 2 wk of daily injections of either native or AGE-modified RSA, aortic tissue from rats receiving AGE-albumin contained nearly 6-fold higher levels of immunoreactive AGEs than animals receiving nonmodified rat albumin or saline vehicle (Fig. 1) ($P < 0.001$). Aortas from animals that received AGE-albumin plus aminoguanidine exhibited less than one-third of the amount of AGE found in animals treated with AGE-albumin alone ($P < 0.05$). Similar results were obtained upon analysis of rabbit aorta samples (data not shown).

Previous studies have indicated that early after its onset, diabetes results in increased vascular permeability (11, 21). In the present studies, the *in vivo* effect of AGEs on endothelial

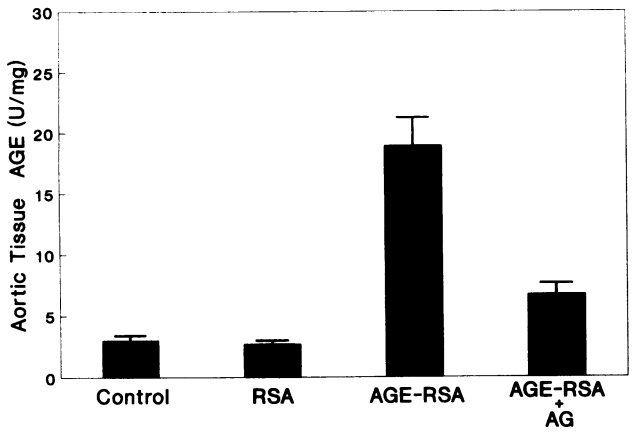

FIG. 1. Normal rat aortic tissue AGE content increases after exogenous administration of AGE-RSA. Following treatment with either AGE-modified RSA (100 mg/kg per day), unmodified RSA (100 mg/kg per day), or AGE-RSA plus aminoguanidine (100 mg/kg per day, i.v.) for 2 wk, AGE levels were determined in collagenase-digested aortic tissue extracts using an AGE-specific ELISA (18). Data are expressed as (means ± SEM) AGE, units/mg of collagen (based on hydroxyproline content), of three independent experiments, each performed in triplicate (n = 5 animals per group). Significant P values: AGE-RSA vs. control, <0.001; AGE-RSA vs. RSA, <0.001; AGE-RSA vs. AGE-RSA plus aminoguanidine (AG), 0.05.

barrier function and permeability was tested in AGE-treated rats at 2 wk, using radioactive tracers. Comparison of the TBIR in organs from the AGE-treated animals with TBIR in corresponding organs from unmodified albumin-treated rats revealed widespread vascular leakage (Fig. 2). The concomitant administration of aminoguanidine completely prevented these increases in tracer permeation in all tissues. Similar observations were made in separate studies, when equivalent amounts of AGE-peptides derived from AGE-RSA by proteinase K digestion were used for injection (data not shown). In those instances, aminoguanidine also prevented the increased vascular permeability response.

An important *in vitro* property of AGEs is their ability to recruit and activate monocytes/macrophages (7, 8). Microscopic examination of tissue sections from rats treated with AGE-RSA for 14 days revealed a significant accumulation of mononuclear cells in the subendothelial and perivascular spaces surrounding the small arteries and arterioles of the liver (Fig. 3*B*), the kidney (Fig. 3*E*), and other tissues, such as skeletal muscle, and subendocardium. The mononuclear cells tended to gather maximally in areas immediately adjacent to small vascular lumens. In the liver, the monocytic infiltration extended further into the sinusoidal spaces (Fig. 3*B*), whereas in the kidney, mononuclear cells appeared around arterioles and peritubular areas (Fig. 3*E*). In contrast, relatively few mononuclear cells were observed in the corresponding tissues from AGE-treated rats also given aminoguanidine (Fig. 3 *C* and *F*). Immunostaining of tissue sections with a macrophage-specific monoclonal antibody revealed numerous positively staining cells (≈50%), suggesting that a significant proportion of this migrating population was either resident macrophages or monocytic in origin (data not shown).

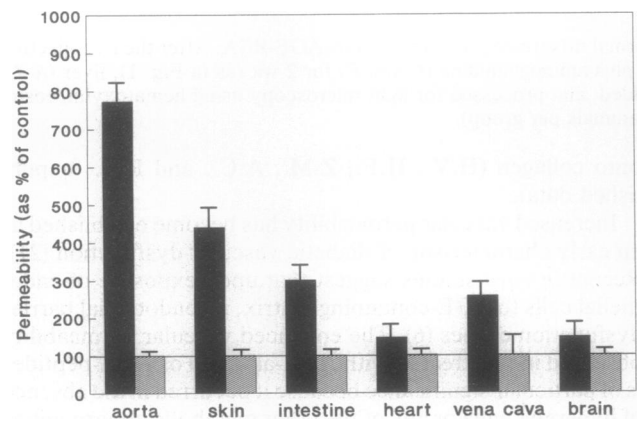

FIG. 2. Exogenous administration of AGEs induces vascular permeability in normal rats. Following treatment with either AGE-RSA, unmodified RSA, or AGE-RSA plus aminoguanidine for 2 wk (as in Fig. 1), tissue permeation by radiolabeled tracers was determined based on TBIR comparisons between AGE-treated (solid bars) or AGE-RSA plus aminoguanidine-treated (hatched bars) and control-unmodified RSA (horizontal line) groups. Data are expressed as percent change of TBIR in tissues of animals treated with AGE or AGE plus aminoguanidine above the TBIR in tissues from control animals treated with unmodified ligand and represent the means ± SEM of six independent experiments (n = 5 animals per group).

Recently, AGEs have been shown to inactivate NO·, an EDRF, via direct chemical reaction (10). We sought to determine the ability of exogenous AGEs to induce a diabetes-like (10, 13) vasodilatory hyporesponsiveness *in vivo* using healthy animals of two different species, rats and rabbits. Mean blood pressure responses to increasing doses of either ACh or NTG in rats pretreated for 2 wk with AGE-albumin were markedly impaired, contrasting with the normal relaxation exhibited by the native albumin-treated and control groups (Fig. 4*A*). Although significant vasodilatory dysfunction was evident by day 10 of AGE administration, peak impairment occurred at day 14 and remained at this severely depressed level for up to 4 wk of AGE-albumin administration (not shown). Similar results were obtained when normal rabbits that were treated for 2 mo with AGE-albumin were studied (Fig. 4*B*). A near-complete blunting of vasodilatory response to either agent was observed in association with AGE treatment, compared to untreated controls or rabbits treated with nonmodified albumin. However, rats or rabbits treated daily with aminoguanidine during the period that they also received AGE-albumin were found to have retained normal vasodilatory responsiveness to either ACh or NTG (Fig. 4).

DISCUSSION

The present observations demonstrate that AGE-modified proteins, when supplied exogenously to otherwise normal animals, exert a range of chemical and biological activities. The data also offer direct *in vivo* confirmatory evidence for a cause and effect relationship between AGE formation and tissue pathology. Using healthy animals, in the absence of glycemia, high tissue AGE levels are shown to be sufficient in and by themselves, to initiate a number of the biochemical, cellular, and pathophysiological abnormalities characteristic of diabetic and aging-related vasculopathy.

Diabetes comprises a complex state of multiple metabolic abnormalities besides hyperglycemia; hence, the *in vivo* role of AGEs in causing diabetic complications directly has remained in question. One approach to this critical issue is to test whether the administration of exogenous AGE-proteins to young, nondiabetic animals can reproduce changes typical of early diabetes-like vascular dysfunction. Despite recent progress, only a few AGEs have been identified in molecular detail and fewer still have been shown definitively to exist *in vivo* (24). We elected to prepare AGE-proteins by incubation *in vitro* of albumin and glucose-6 phosphate to produce a mixture of intermediate and late AGEs that is believed to represent the diverse spectrum of adducts that exists *in vivo*. Consistent with this, the *in vitro*-prepared AGEs used in

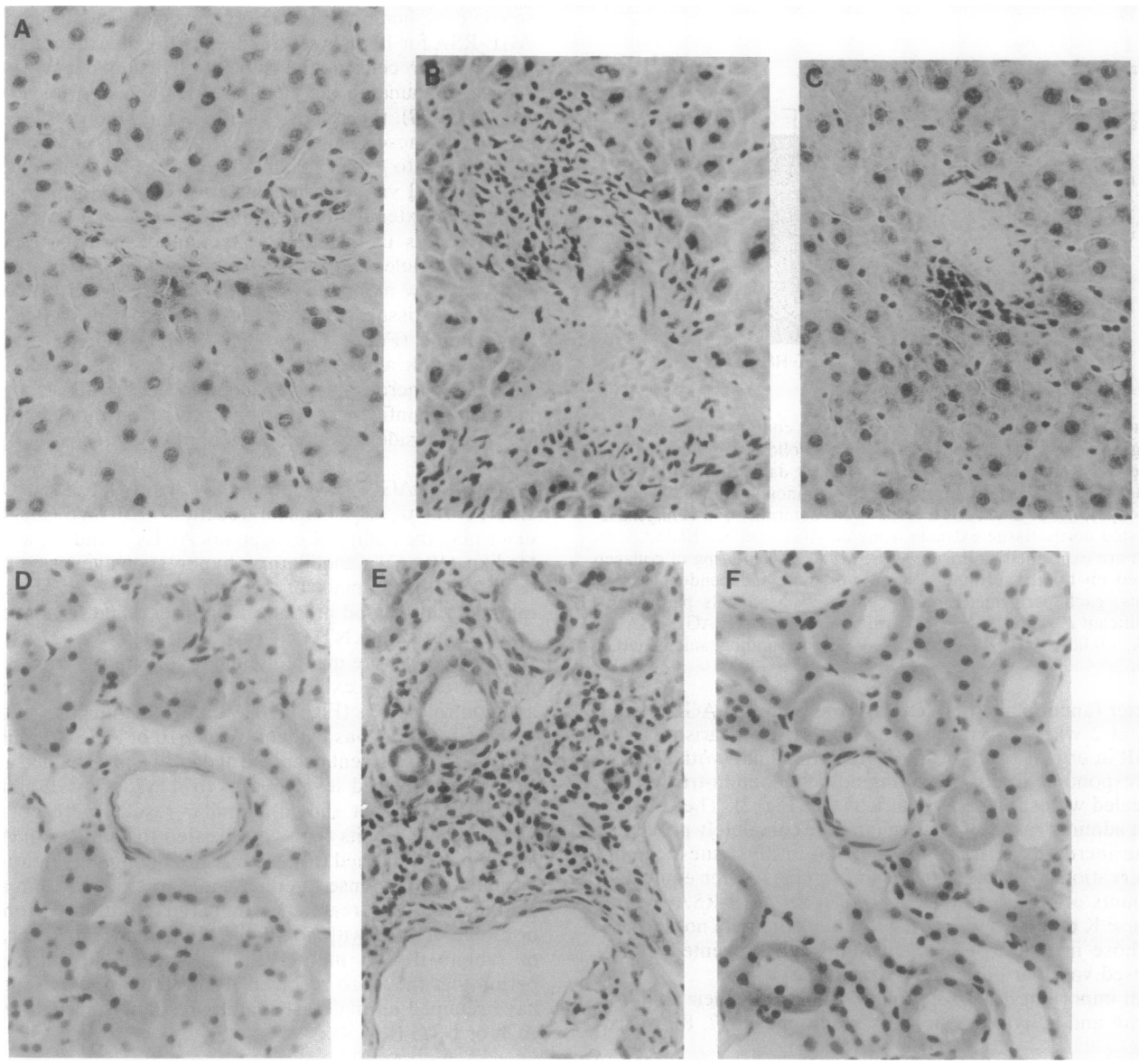

FIG. 3. Increased mononuclear cell migratory activity in tissues from normal rats treated with exogenous AGE-RSA. After the i.v. injection of either unmodified RSA (*A* and *D*), AGE-RSA (*B* and *E*), or AGE-RSA plus aminoguanidine (*C* and *F*) for 2 wk (as in Fig. 1), liver (*A–C*) and kidney (*D–F*) tissues were fixed in 10% formaldehyde, paraffin-embedded, and processed for light microscopy using hematoxylin/eosin. (×250.) Data are representative of three independent experiments (n = 3 animals per group).

these experiments cross-react with AGE-specific antibodies that recognize *in vivo*-formed AGEs (18).

AGE-albumin infusion resulted in the covalent attachment of a significant amount of AGEs within the extracellular matrix of rat and rabbit aorta, found to be quantitatively similar to the levels seen in diabetic human aortas (19). This attachment was inhibited by the crosslinking inhibitor aminoguanidine, providing evidence of the covalent nature of AGE deposition.

Intact molecules of exogenous AGE-albumin may react directly with tissue matrix proteins via available AGE crosslinking moieties (25). Alternatively, exogenous AGE-albumin may undergo partial proteolytic digestion and denaturation, thus exposing additional reactive AGEs, further facilitating protein crosslinking. Such AGE "reactivity" may be particularly important in those patients with diabetic nephropathy and end-stage renal disease (ESRD) in whom circulating AGEs are markedly elevated (19). Serum AGE-peptides when isolated from diabetic patients with ESRD have been found to be highly reactive, rapidly crosslinking onto collagen (H.V., H.F., Z.M., A.C., and R.B., unpublished data).

Increased vascular permeability has become established as an early characteristic of diabetic vascular dysfunction (21). Recent *in vitro* studies suggest that upon exposure of endothelial cells to AGE-containing matrix, an endothelial barrier dysfunction ensues (6). The enhanced vascular permeability observed in rats treated with AGE-albumin or AGE-peptides is of particular significance because it occurred in the absence of hyperglycemia or any of the other metabolic abnormalities that occur with diabetes. The conclusion that AGE-peptide attachment occurs prior to the induction of vascular leakiness was reinforced by the complete prevention of this dysfunction with aminoguanidine treatment. Although AGE-mediated leakiness may result from several distinct mechanisms, such as endothelial cell dysfunction (6) and the release of macrophage-derived cytokines (8), and other regulatory molecules such as EDRF, the present study suggests that covalent AGE attachment is necessary to initiate this process *in vivo*.

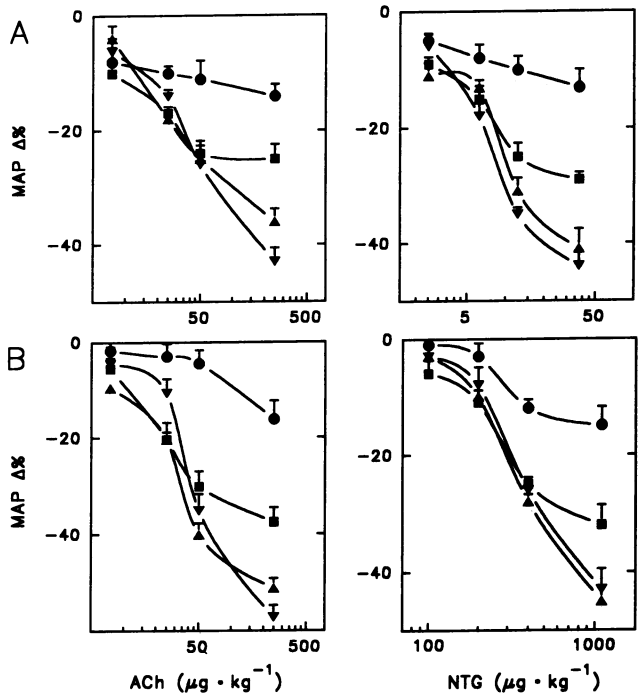

FIG. 4. Vasodilatory impairment in healthy rats (*A*) and rabbits (*B*) following administration of AGE-modified albumin. After a period of daily injections with species-matched AGE-albumin (circles), AGE-albumin plus aminoguanidine (squares), or unmodified albumin (triangles), vasodilatory responses to increasing doses of ACh or NTG were compared to untreated controls (inverted triangles). MAP, mean arterial pressure. Data represent the means ± SEM obtained from eight rats (*A*) and four rabbits (*B*). Significant *P* values: AGE-treated vs. untreated control animals, <0.001; AGE vs. AGE plus aminoguanidine, <0.001 for either *A* or *B* and for either drug, ACh or NTG.

The deposition of AGEs in otherwise normal subendothelial and perivascular spaces was accompanied by significant mononuclear cell infiltration. This is consistent with the previously reported *in vitro* chemotactic activity of AGE-modified soluble and matrix proteins, powerful enough to promote monocyte migration through an intact endothelial monolayer *in vitro* (7). Since the early stages of atherogenesis are characterized by monocytic infiltration (26), vessel wall AGE-induced chemotaxis may be an early initiating event in diabetic vasculopathy.

The initial size of the recruited cell population at sites of maximal aggregation, which is probably due to the rapid deposition of large amounts of exogenous AGE within the tissues, decreases as a function of time after cessation of AGE administration, presumably by effective phagocytosis (unpublished observations). Regardless of the precise mechanistic differences between "acute" *in vivo* AGE deposition achieved in these studies and the progressive AGE formation that occurs in diabetes over many years, the preventive influence of aminoguanidine on monocytic infiltration confirms the specificity of an AGE-mediated monocytic response.

It has been long known that AGE-mediated alterations of the physicochemical properties of collagen—e.g., loss of elasticity, increased protein crosslinking and thickness, etc.—are likely to influence vascular function and tone. Recent observations have revealed that AGEs chemically inactivate NO· and that AGE formation within the diabetic vessel wall modulates NO·-dependent vascular relaxation (10). In the present studies, exogenous AGEs were found to induce marked inhibition of vasodilatory response in normal animals that was prevented to a significant extent by aminoguanidine treatment (10). These results are consistent with an AGE-mediated NO· quenching effect, dependent largely on the covalent attachment of AGE moieties within the vascular matrix.

Taken together, these observations provide direct evidence for the pathogenic properties of AGEs in an *in vivo* system that dissociates AGE accumulation from the metabolic, hormonal, and/or genetic determinants associated with diabetes or with normal aging.

These studies introduce a valuable model for studying the direct pathogenicity of circulating AGEs, which occur at elevated levels in normoglycemic patients with ESRD and are particularly high in those patients with diabetic ESRD (19). Circulating AGEs appear to represent an important class of "uremic toxins" responsible for progressive vascular disease and rapid clinical deterioration in diabetic patients with ESRD (27). These results also emphasize the therapeutic potential of AGE-crosslink inhibitors, such as aminoguanidine, in preventing the complex vascular dysfunction of diabetes mellitus.

We thank Dr. Kirk Manogue for carefully reviewing the manuscript and Donna Bovè for excellent editorial assistance. This work was supported, in part, by National Institutes of Health Grants AG-9453 to H.V. and DK-19655 to A.C. and by a grant from the American Diabetes Association to R.B.

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